Published in final edited form as:

Mol Nutr Food Res. 2016 April ; 60(4): 858–870. doi:10.1002/mnfr.201500814.

Green Tea Extract Provides Extensive Nrf2-Independent Protection Against Lipid Accumulation and NFκB Pro-Inflammatory Responses During Nonalcoholic Steatohepatitis In Mice Fed A High-Fat Diet

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Abstract

Scope—Green tea extract (GTE) reduces liver steatosis and inflammation during nonalcoholic steatohepatitis (NASH). We hypothesized GTE would mitigate NASH in a nuclear factor erythroid-2-related-factor-2 (Nrf2)-dependent manner in a high fat (HF)-induced model.

Methods and results—Nrf2-null and wild-type (WT) mice were fed a HF diet containing 0 or 2% GTE for 8 wk prior to assessing parameters of NASH. Compared to WT mice, Nrf2-null mice had increased serum alanine aminotransferase, hepatic triglyceride, expression of free fatty acid uptake and lipogenic genes, malondialdehyde, and NFκB phosphorylation and expression of pro-inflammatory genes. In WT mice, GTE increased Nrf2 and NADPH:quinone oxidoreductase-1 mRNA, and lowered hepatic steatosis, lipid uptake and lipogenic gene expression, malondialdehyde, and NFκB-dependent inflammation. In Nrf2-null mice, GTE lowered NFκB phosphorylation and TNFα and MCP1 mRNA to levels observed in WT mice fed GTE whereas hepatic triglyceride and lipogenic genes were lowered only to those of WT mice fed no GTE. Malondialdehyde was lowered in Nrf2-null mice fed GTE, but not to levels of WT mice, and without improving the hepatic antioxidants α-tocopherol, ascorbic acid, and uric acid.

Conclusion—Nrf2 deficiency exacerbates NASH whereas anti-inflammatory and hypolipidemic activities of GTE likely occur largely independent of Nrf2 signaling.

Graphical abstract

Green tea extract (GTE) protects against nonalcoholic steatohepatitis (NASH) through hypolipidemic and antiinflammatory activities. In wild-type and Nrf2-null mice that were fed a high-fat diet with or without GTE for 8 wk, Nrf2 deficient mice had an exacerbated pathology of

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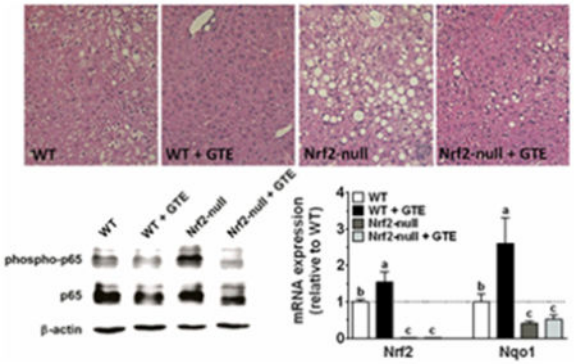
The authors have declared no conflict of interest.

NASH including greater lipid accumulation and NFκB-dependent inflammatory responses. GTE mitigated liver steatosis and inflammation in wild-type mice while also increasing Nrf2 and Nqo1 expression. However, GTE also significantly lowered NFκB phosphorylation and liver steatosis in Nrf2-null mice. Thus, GTE provided extensive Nrf2-independent hepatoprotection to limit liver injury and inflammation during NASH.



Keywords

green tea; NASH; Nrf2; oxidative stress; inflammation

1 Introduction

Nonalcoholic fatty liver disease (NAFLD) is the most common liver disorder in the United States [1]. It is a progressive disorder that advances in severity from liver steatosis to nonalcoholic steatohepatitis (NASH), fibrosis, and cirrhosis to increase hepatocellular carcinoma risk [2]. An estimated 70 million Americans have NAFLD [3], and its prevalence is expected to increase due to its close association with obesity and diabetes [4] and a lack of validated therapies that mitigate its development and progression.

The pathogenesis from steatosis to NASH is often described by the “two-hit” mechanism [5]. The “first-hit”, mediated by obesity, insulin resistance, and dysregulated lipid metabolism, results in excess hepatic lipid accumulation [5]. Consequently, steatotic livers are vulnerable to “second-hits”, mediated by oxidative stress and inflammation, that exacerbate liver injury [5]. The involvement of reactive oxygen species (ROS) during NASH [5] supports a role of nuclear factor erythroid-2-related factor-2 (Nrf2) in limiting oxidative stress that otherwise induces liver injury [6]. Indeed, transcriptional activities of Nrf2 regulate xenobiotic metabolism and antioxidant defenses [6]. They also reduce inflammation and fatty acid synthesis [7-10], suggesting that its cytoprotective activities could attenuate NASH through multiple mechanisms.

Nrf2-dependent hepatoprotection has been demonstrated in several models of NASH. Nrf2-null mice fed a methionine- and choline-deficient (MCD) diet had exacerbated pathology of NASH, including greater expression of pro-inflammatory and fatty acid metabolism genes as well as histological and biochemical markers of liver injury and lipid peroxidation [11]. Conversely, Kelch-like ECH-associated protein 1 (Keap1) knockout mice fed a MCD diet

are protected from NASH due to their constitutive activation of Nrf2 [12]. Nrf2-null mice fed a high-fat (HF) diet also show that Nrf2 reduces steatosis and oxidative stress by suppressing lipogenic and cholesterogenic pathways [13]. Nrf2 may also mitigate oxidative stress during NASH by improving redox status, consistent with its role in regulating glutathione biosynthesis and expression of antioxidant defenses (e.g. NADPH:quinone oxidoreductase 1 (Nqo1)) [6].

NASH patients and rodent models of NASH have greater nuclear factor κB (NFκB) activation and expression of pro-inflammatory genes including tumor necrosis factor-α (TNFα) and monocyte chemoattractant protein-1 (MCP-1) [14, 15]. NFκB activation occurs through intracellular and extracellular pathways that converge at the phosphorylation of IκB kinase (IKK), which promotes IκB phosphorylation and nuclear translocation of NFκB [16]. Nrf2 deficiency increases NFκB activation during NASH [17], consistent with evidence from a pro-inflammatory experimental model that Nrf2 activation decreases IKK activity and phosphorylation and degradation of IκBα [18]. Thus, therapies that induce Nrf2 would be expected to attenuate NFκB-dependent pro-inflammatory responses otherwise promoting liver injury during NASH.

High dietary intakes of green tea are associated with a lower risk of liver injury, hyperlipidemia, and inflammation [19]. Although no clinical studies have examined green tea during NASH, green tea extract (GTE) protects against NASH, likely through its major catechin epigallocatechin gallate (EGCG), in genetic and diet-induced models through hypolipidemic, antioxidant, and anti-inflammatory mechanisms [20]. For example, GTE attenuated NASH in genetically obese mice by lowering lipogenic gene expression and reducing lipid peroxidation in association with an upregulation in enzymatic activities of several Nrf2-dependent enzymatic antioxidant defenses that detoxify ROS [21]. In a dietary HF-induced model of NASH, GTE lowered NFκB binding activity by reducing IκBα phosphorylation while also decreasing protein and mRNA levels of TNFα and MCP-1 [22]. Consistent with these findings and evidence that GTE improves endogenous antioxidant defenses under the regulation of Nrf2 [21], we hypothesized in the present study that hepatoprotective activities of GTE during NASH are mediated in a Nrf2-dependent manner. We therefore fed Nrf2-null mice and their wild-type (WT) controls a HF diet containing GTE at 0 or 2%, and then assessed markers of liver injury and oxidative stress, expression of genes involved in fatty acid uptake and lipogenesis, and NFκB-dependent inflammatory responses to define Nrf2-dependent hypolipidemic and anti-inflammatory activities of GTE during NASH.

2 Materials and Methods

2.1 Materials

All solvents were HPLC-grade or higher and were purchased from Thermo Scientific (Waltham, MA) along with the following chemicals: citric acid, EDTA, and PBS. BSA, EGCG, thiobarbituric acid (TBA), Tris-buffered saline with Tween-20 (TBST), and 1,1,3,3-tetramethoxypropane (TPP) were from Sigma (St. Louis, MO).

2.2 In Vitro Study

HC-04 human hepatocytes, a cell line well-suited for biomechanistic studies of xenobiotics [23], were used to examine dose- and time-dependent effects of EGCG, the predominant polyphenolic catechin in green tea [20], on Nrf2 activation and expression. HC-04 cells were obtained from BEI Resources (American Type Culture Collection; Manassas, VA). In all experiments, cells were incubated (5% CO_2; 37°C) in low glucose Dulbecco's Modified Eagle Medium (DMEM; Sigma) supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic (100 U/mL penicillin G sodium, 100 μg/mL streptomycin sulfate, and 0.25 μg/mL amphotericin B). EGCG at physiologically relevant concentrations (0-5 μM) [20] was dissolved in 0.05% citric acid and all experiments were performed in serum-free DMEM containing 1% antibiotic-antimycotic. Cells were seeded at a density of 4×10^5 for mRNA expression studies or 8×10^5 for all other studies and were harvested at specific time points up to 2 h following incubation with EGCG. Nuclear accumulation of Nrf2 protein in response to EGCG (0-5 μM) or the pro-oxidant positive control t-butylhydroperoxide (100 μM) was assessed by Western Blot, as described below, after isolating the nuclear fraction using a kit (Active Motif, Carlsbad, CA). Nrf2 mRNA expression was assessed at 0-5 μM EGCG at 0-2 h post-treatment using RT-PCR, as described below, after total RNA was isolated using Trizol (Invitrogen, Carlsbad, CA). Cytotoxicity was assessed using the lactate dehydrogenase (LDH) assay (Sigma) and the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS; Promega, Madison, WI) assay at 2 h post-treatment according to the manufacturers' instructions.

2.3 Mouse Study

The protocol for this study was approved by the Institutional Animal Care and Use Committee (2012A00000156) at The Ohio State University. Homozygous Nrf2-null mice on a C57BL/6 background provided by Dr. Angela Slitt (University of Rhode Island) were bred at University of Connecticut, and then male Nrf2-null mice (n = 23) were supplied to The Ohio State University. Male C57BL6 WT mice (n = 20) were purchased from Charles River Laboratories (Wilmington, MA). All mice were acclimated to the temperature-, light-, and humidity-controlled facility for at least 2 wk until WT and Nrf2-null mice were 11-12 wk old. Nrf2-null and WT mice were housed individually while receiving a HF diet containing powdered GTE at 0 or 2% (w/w) for 8 wk. The HF diet containing 60% of energy from fat (primarily as lard), as detailed previously [22, 24], was formulated to contain 0 or 2% GTE, which was kindly provided by Unilever BestFoods.

Powdered GTE contained 30% total catechins (w/w), which consisted of 48% EGCG, 31% epigallocatechin, 13% epicatechin gallate, and 8% epicatechin as verified by HPLC-UV [25]. GTE at 2% was chosen based on our studies showing that it reduces liver steatosis and NFκB-dependent inflammation in genetic and HF-induced models of NASH [21, 22, 25]. GTE at 2% is also closely equivalent to 10 servings/d (120 mL/serving) in humans [25], a dietary level associated with a lower risk of liver injury in Japanese adults [19]. Mice had free access to food and water throughout the study. Body mass was measured weekly and food intake daily. After the feeding period, mice were sacrificed in the fasted state (10-12 h) under isoflurane. Blood was collected from the retroorbital sinus and centrifuged to obtain serum. Tissues were excised, rinsed in PBS, blotted, and frozen in liquid nitrogen before

storing at -80°C. A portion of the central hepatic lobe was collected into formalin for histological assessment or into RNAlater (Sigma) for gene expression studies.

2.4 Liver Histology

Paraffin-embedded liver sections (4-5 μm) were stained with hematoxylin and eosin. Images were captured using an Olympus IX50 microscope from 10 randomly selected fields (200× magnification) to assess NASH using established histologic criteria that score steatosis, hepatocellular ballooning, and lobular inflammation [26]. In brief, steatosis was scored as: grade 0 for no fatty hepatocytes, grade 1 for fatty hepatocytes occupying <33% of the hepatic parenchyma, grade 2 for fatty hepatocytes occupying 33-66% of the hepatic parenchyma, or grade 3 for fatty hepatocytes occupying >66% of the hepatic parenchyma. Hepatocellular ballooning was scored as grade 0 for none, grade 1 for few ballooned cells, and grade 2 for predominant ballooning. Hepatic inflammatory infiltrates were scored as grade 0 for none, grade 1 for <2 foci/field, grade 2 for 2-4 foci/field, or grade 3 for >4 foci/field.

2.5 Lipids and Clinical Assays

Hepatic total lipid was determined gravimetrically [25], and solubilized to determine triglyceride, cholesterol (Pointe Scientific, Canton, MI) and nonesterified fatty acid (NEFA; Wako Diagnostics, Mountain View, CA) using spectrophotometric kits. Serum lipids were determined directly with these kits. Serum glucose was measured by clinical assay (Pointe Scientific) and insulin by ELISA (Crystal Chem, Downers Grove, IL). Serum alanine aminotransferase (ALT) activity was measured by clinical assay (Pointe Scientific).

2.6 RT-PCR

Gene expression studies were performed as described [21], with minor modifications. In brief, total RNA was extracted using TRIzol (Invitrogen). RT-PCR was performed using a SYBR Green PCR Kit, a Bio-Rad CFX384 instrument (Hercules, CA), and primer sequences purchased from Sigma (Table 1). mRNA expression of human Nrf2 in HC-04 cells were quantified relative to β-actin using the $2^{-\Delta\Delta CT}$ method [27]. For studies in mice, hepatic expression of genes involved in antioxidant defense [Nrf2; Nqo1], lipid metabolism [cluster of differentiation-36 (CD36); sterol regulatory element binding protein-1c (SREBP-1c); fatty acid synthase (FAS); stearoyl-CoA desaturase-1 (SCD1); diglyceride acyltransferase (DGAT1/2)] and inflammation [MCP-1, TNFα, inducible nitric oxide synthase (iNOS); toll-like receptor-4 (TLR4); tumor necrosis factor receptor-1 (TNFR1)] were quantified relative to hypoxanthine-guanine phosphoribosyl transferase (HPRT).

2.7 Western Blotting

Total cellular proteins were extracted from liver using a Pierce T-PER kit containing Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific). Proteins were denatured under reducing conditions, separated on a 10% SDS-PAGE, and transferred to a nitrocellulose membrane. For studies in HC-04 cells, membranes were probed overnight with primary antibodies against Nrf2 (#12721; Cell Signaling Technology; Danvers, MA) or TFIIB (#SC-225; Santa Cruz Biotechnology; Dallas, Texas) that were diluted 1:2000 in 2%

non-fat dried milk in TBST. For studies in mice, membranes were probed for 1 h at room temperature with primary antibody against β-actin (#4967; Cell Signaling Technology) that was diluted 1:1000 in TBST, or they were probed overnight at 4°C with antibodies against phosphorylated-p65 (phospho-p65; 1:500; #3033) and p65 (1:1000; #8242) prepared in TBST containing 5% (w/v) BSA. After washing in TBST, membranes were incubated with horseradish peroxidase-conjugated secondary antibody (1:1000; #SC-2056; Santa Cruz Biotechnology). Detection was accomplished by enhanced chemiluminescence and densitometry analysis on a Kodak 2000RT imaging station using 1D software.

2.8 Hepatic Antioxidants and Lipid Peroxidation

Hepatic ascorbic acid and uric acid were measured by HPLC as described [25], with minor modification to use an UltiMate 3000 HPLC system (Thermo Scientific) equipped with two 6011RS coulometric cells programmed to 25, 150, 250, and 350 mV. Hepatic α-tocopherol was measured by LC-MS as we described [28] following saponification and hexane extraction. Hepatic malondialdehyde (MDA) was extracted and measured by HPLC-FL as described [21], with minor modifications. In brief, liver was homogenized in lysis buffer (Gold Biotechnology; St. Louis, MO) containing 5 mM DL-dithiothreitol (Sigma), 5 mM EDTA disodium salt, and protease and phosphatase inhibitor cocktail (Thermo Scientific). Following centrifugation, the supernatant was mixed with TBA, incubated, and the butanol extract was injected on a HPLC-FL system set to 532/553 nm (excitation/emission). Separation was performed at 1 mL/min on an Alltima C18 column (250 × 4.6 mm, 5 μm; Columbia, MD) using 50:50 methanol and phosphate buffer (pH 6.5). MDA was quantified from standards prepared in parallel from TPP, and normalized to hepatic protein.

2.9 Statistical Analysis

Data (means ± SEM) were analyzed using GraphPad Prism, version 6 (GraphPad Software; La Jolla, CA). 1-way or 2-way ANOVA was used as appropriate for studies performed in HC-04 cells. For mouse studies, 2-way ANOVA was used to evaluate main effects due to genotype and GTE, and their interaction. Variables with unequal variance were log-transformed to achieve a normal distribution. Newman-Keuls post-hoc test was used to evaluate group mean differences following statistically significant main or interactive effects as appropriate. Groups without a common superscript were statistically significant different at $P \leq 0.05$.

3 Results

3.1 EGCG increases nuclear Nrf2 accumulation in vitro and dietary GTE increases hepatic Nrf2 expression

Studies in HC-04 cells were conducted to define the effect of physiologic concentrations of EGCG on nuclear accumulation of Nrf2. Western blotting studies indicated that 2 h treatment with EGCG (0.1-5 μM) increased nuclear accumulation of Nrf2 at all concentrations tested (Figure 1A). This occurred without any time (0-2 h)- or concentration (0-5 μM)-dependent changes in Nrf2 mRNA expression (Figure 1B). Increases in nuclear Nrf2 accumulation by EGCG also occurred without affecting cytotoxicity (Figure 1C-D). Further corroborating, livers of mice chronically fed a HF diet containing GTE showed

increased ($P<0.05$) mRNA expression of Nrf2 and its transcriptional target Nqo1 compared to mice fed the HF diet containing no GTE (Figure 2). Expression of these genes in Nrf2-null mice were lower than WT mice regardless of GTE. Collectively, data support that hepatoprotective activities of green tea observed in rodent models of NASH [20] may be mediated through Nrf2.

3.2. GTE reduces body mass independent of food intake and Nrf2 status, but reductions in adiposity by GTE are partly Nrf2-dependent

At study onset, body mass was not different between Nrf2-null and WT mice (Table 2). Following the 8 wk feeding period, body mass of Nrf2-null mice was lower than those of WT mice despite no significant difference in food intake ($P>0.05$). Body masses of WT and Nrf2-null mice fed GTE were lower than those of their respective controls, but without any genotype differences. Liver and adipose mass did not differ between Nrf2-null and WT mice. However, GTE lowered liver mass to a similar extent regardless of genotype, whereas adiposity was lowered by GTE to a greater extent in WT mice compared to Nrf2-null mice (Table 2).

3.3 GTE reduces hepatic steatosis and injury regardless of Nrf2 deficiency

Histologic evaluation provided clear visual evidence that Nrf2-null mice had exacerbated liver steatosis and hepatocellular ballooning compared to WT mice (Figure 3). Although GTE reduced these parameters in both genotypes, their reduction was more pronounced in WT mice fed GTE in which there was little or no histologic evidence of steatosis or ballooning. In contrast, inflammatory infiltration was minimal regardless of genotype or GTE.

Hepatic lipid was quantified to corroborate histologic evidence of steatosis (Table 2). A genotype × GTE interaction ($P<0.05$) indicated that hepatic total lipid was increased in Nrf2-null mice compared with WT mice, but that GTE reduced it to lower levels in WT mice compared to Nrf2-null mice. Higher hepatic total lipid in Nrf2-null mice was attributed to greater hepatic triglyceride and cholesterol (Table 2). However, hepatic cholesterol within each genotype was unaffected by GTE, whereas hepatic triglyceride was lowered by GTE regardless of genotype, and more greatly lowered in WT mice fed GTE. Hepatic NEFA was unaffected by Nrf2 or GTE.

Serum ALT activity, a biomarker of liver injury, was greater in Nrf2-null mice compared to WT mice (Table 2). Serum ALT was lower in WT and Nrf2-null mice fed GTE compared to their respective controls with no difference in ALT between WT and Nrf2-null mice fed GTE. Serum glucose was unaffected by genotype, but was reduced to a similar extent by GTE in both WT and Nrf2-null mice (Table 2). Serum insulin was higher in Nrf2-null mice compared to WT mice. GTE lowered insulin levels in both genotypes relative to respective controls with no differences between WT and Nrf2-null mice fed GTE. Lastly, serum NEFA was unaffected in Nrf2-null mice, but was lowered by GTE in WT mice only (Table 2). Together, a HF diet exacerbates liver steatosis and injury, and insulinemia in Nrf2-null mice, but GTE-mediated improvements in these parameters occurred largely independent of Nrf2 status.

3.4 GTE lowers mRNA expression of hepatic lipid uptake and lipogenic genes regardless of Nrf2 status

To assess hepatic lipid-lowering activities of GTE, expression of genes involved in free fatty acid uptake and lipogenesis were examined (Figure 4). Nrf2-null mice compared with WT mice had increased mRNA of the fatty acid uptake transporter CD36 as well as the lipogenic transcription factor SREBP-1c and its downstream targets FAS and SCD1. Nrf2-null mice also had greater DGAT-1 and -2 mRNA compared with WT mice. Although GTE reduced expression of each gene, GTE lowered their expression to a greater extent in WT mice fed GTE compared to Nrf2-null mice fed GTE with the exception of DGAT-1 and -2, which were reduced to a similar extent by GTE in both genotypes. Thus, liver steatosis in Nrf2-null mice is likely exacerbated by greater hepatic uptake of free fatty acids and lipid synthesis, but GTE likely only provides limited Nrf2-dependent hepatoprotection on these parameters.

3.5 GTE lowers lipid peroxidation otherwise increased by Nrf2 deficiency without improving antioxidant status

Consistent with greater liver steatosis (Figure 2), Nrf2-null mice had greater hepatic MDA compared to WT controls (Figure 5A). Although MDA was significantly lower in both WT and Nrf2-null mice fed GTE compared to their respective controls, MDA in Nrf2-null mice fed GTE remained significantly higher than those of WT mice fed no GTE (Figure 5A). Endogenous and dietary antioxidants known to regulate oxidative stress were then assessed to better define the interaction of GTE and Nrf2 status on hepatic lipid peroxidation. Ascorbic acid, which is devoid from the purified diet because rodents synthesize it, was lower in Nrf2-null mice and unaffected by GTE regardless of genotype (Figure 5B). Uric acid, a byproduct of purine catabolism that has antioxidant activity at physiologic levels [29], was lower in Nrf2-null mice and unaffected by GTE in both WT and Nrf2-null mice (Figure 5C). To the contrary, Nrf2-null mice had greater accumulation of hepatic α-tocopherol compared to WT mice (Figure 5D). Nrf2-null mice fed GTE had α-tocopherol levels lowered to the extent of WT controls fed no GTE, whereas α-tocopherol of WT mice fed GTE was lower than that of WT mice. However, normalization of α-tocopherol to total hepatic lipid resulted in Nrf2-null mice having greater α-tocopherol, but without any effect of GTE in either genotype (data not shown). Collectively, Nrf2 deficiency exacerbates hepatic MDA, but GTE-mediated improvements in lipid peroxidation occur without improving hepatic antioxidants.

3.6 GTE reduces inflammatory responses otherwise increased by Nrf2 deficiency

NFκB activation increases with oxidative stress and contributes to hepatic injury during NASH [22]. Consistent with GTE reducing NFκB-dependent inflammatory responses during NASH in association with lowered oxidative stress [21, 22], we hypothesized that anti-inflammatory activities of GTE would occur in a Nrf2-dependent manner. Consistent with our hypothesis, Nrf2-null mice had greater protein levels of phospho-p65, a transcriptionally active form of NFκB [30], compared with WT mice (Figure 6). Phospho-p65 levels were lowered in WT mice fed GTE. However, and contrary to our hypothesis, GTE also lowered phospho-p65 levels in Nrf2-null mice to the extent observed in WT mice fed GTE. These

decreases in phospho-p65 occurred without any Nrf2- or GTE-mediated alterations in total p65 protein (Figure 6).

To corroborate the opposing activities of Nrf2 status and GTE on NFκB activation, expression of NFκB-dependent target genes implicated in NASH were assessed. Consistent with Nrf2-null mice having increased phospho-p65 protein, they also had greater TNFα, MCP-1, and iNOS mRNA compared with WT mice (Figure 7). GTE lowered mRNA levels of each of these pro-inflammatory targets regardless of genotype. However, iNOS mRNA in Nrf2-null mice fed GTE was only lowered to the extent observed in WT mice, whereas TNFα and MCP-1 were lowered to levels not different from WT mice fed GTE. Thus, while Nrf2 deficiency increases NFκB-dependent inflammation, anti-inflammatory activities of GTE during NASH appear to be mediated largely in a Nrf2-independent manner. Consistent with this notion, we considered an alternative hypothesis that anti-inflammatory activities of GTE could be mediated by cellular receptors that trigger NFκB activation during NASH, specifically TNFR1 and TLR4 [31, 32]. Indeed, hepatic mRNA expression of both TNFR1 and TLR4 was higher in Nrf2-null mice compared with WT mice (Figure 8). GTE reduced TNFR1 and TLR4 mRNA expression regardless of genotype with no difference in mRNA levels between genotypes.

4 Discussion

Consistent with our hypothesis, and the work of others [6], Nrf2 deficient mice fed a HF diet had exacerbated NASH pathology as evidenced by greater NFκB-dependent pro-inflammatory responses, along with increased hepatic lipid peroxidation, liver steatosis, and expression of hepatic free fatty acid uptake and lipogenic genes. Contrary to our hypothesis, and despite evidence suggesting Nrf2-mediated hepatoprotective activities of GTE during NASH [21, 22] that are likely mediated through EGCG [20]. Our data suggest that GTE provides limited Nrf2-dependent protection against liver steatosis and injury, lipogenic gene expression, and activation of NFκB and expression of its pro-inflammatory mediators. In addition, although GTE lowers hepatic lipid peroxidation regardless of Nrf2 status, this occurred without any GTE-mediated improvements in hepatic antioxidants in Nrf2-null mice. Collectively, anti-inflammatory and hypolipidemic activities of GTE during NASH likely occur largely through a mechanism independent of Nrf2 activation.

High dietary green tea intakes (≥10 servings/d) are associated with lower biomarker levels of liver injury, inflammation, and hyperlipidemia [19]. In agreement, evidence in genetic and diet-induced rodent models of NASH support that dietary GTE or its principal catechin EGCG reduces liver steatosis and injury, insulin resistance, oxidative stress, and inflammation [20]. Although the mechanism underlying these health benefits remains unclear, hepatoprotective mechanisms of GTE likely occur through both Nrf2-independent and -dependent mechanisms. For example, EGCG reduces intestinal lipid absorption by inhibiting pancreatic phospholipase A_2 activity [33] and may mitigate insulin resistance by reducing postprandial glucose resulting from starch ingestion by inhibiting α-amylase activity [34]. In contrast, studies in *ob/ob* mice and rats fed a HF diet support a Nrf2-dependent protective mechanism consistent with GTE upregulating enzymatic activities of antioxidant defenses under the transcriptional control of Nrf2 (e.g. glutathione *S*-transferase,

glutathione peroxidase, catalase) [21]. GTE also reduced NFκB activation and expression of TNFα and MCP-1 that were otherwise increased by a HF diet, and their lowering occurred in association with improved glutathione status [22], consistent with studies in *ob/ob* mice showing that GTE increases the expression of Nrf2-dependent genes regulating glutathione biosynthesis [21].

Supporting our hypothesis that GTE would protect against NASH in a Nrf2-dependent manner, we show that physiologically relevant concentrations (0.1-5 μM) of EGCG [20], a prominent constituent of GTE, increase nuclear protein accumulation of Nrf2 in HC-04 cells in agreement with studies *in vitro* and in rodent models indicating EGCG-mediated activation of Nrf2-dependent targets that detoxify ROS [35]. This cytoprotective response is thought to be mediated through the electrophilic potential of EGCG [35], which is readily auto-oxidized at near-neutral pH similar to that occurring during digestion, and absorbed by Caco-2 cells [36]. The EGCG electrophile is then expected to promote oxidative modification of cysteine residues on Keap1. This induces conformation changes of the Keap1-Nrf2 complex to suppress Nrf2 degradation by limiting ubiquitination, and facilitates nuclear translocation of newly synthesized Nrf2 where it binds to antioxidant response elements (ARE) to regulate the expression of antioxidant and xenobiotic defenses [37, 38]. Consistent with these findings, we show that GTE in WT mice fed a HF diet, but not in Nrf2-null mice, increases mRNA expression of Nrf2 and its downstream target gene Nqo1, which assists in lowering oxidative stress by maintaining cellular antioxidants in their reduced form, scavenging superoxide, and catalyzing reductive metabolism of xenobiotics [6]. Although numerous antioxidant defenses largely regulated by Nrf2 are also regulated by other transcription factors [39], the present study aimed to define the extent to which antioxidant activities of GTE protect against NASH in a Nrf2-dependent manner by lowering NFκB-dependent inflammation and hepatic lipid accumulation, which have been shown to be attenuated by dietary GTE or EGCG in obese models of NASH [21, 22, 24, 25, 40, 41].

NFκB can be activated in a redox-dependent manner by oxidative stress. In agreement, Nrf2-null mice fed a MCD diet to induce NASH have greater lipid peroxidation along with nuclear accumulation of the p65 subunit of NFκB and mRNA expression of its pro-inflammatory mediators [17]. In our study, livers of Nrf2-null mice fed a HF diet have increased phospho-p65 protein and TNFα, MCP-1, and iNOS mRNA expression along with higher MDA compared to WT controls. GTE lowered hepatic NFκB binding activity in rats fed a HF diet [22]. This is corroborated in our study by GTE-mediated decreases in phospho-p65 protein accumulation and TNFα and MCP-1 mRNA expression in WT mice, consistent with EGCG reducing circulating MCP-1 in mice fed a HF diet [24]. However, these inflammatory responses in Nrf2-null mice fed GTE were also lowered, and to levels no different from WT mice fed GTE, suggesting that anti-inflammatory activities of GTE occur in a Nrf2-independent manner. Furthermore, the hepatic antioxidants ascorbic acid and uric acid, a byproduct of purine metabolism known to be regulated by Nrf2 [42], were also lowered by Nrf2 deficiency, and otherwise unaffected by GTE regardless of Nrf2 status.

Insulin resistance is an early event leading to NASH [5], although direct evidence also shows that TNFα-mediated inflammation accelerates the induction of liver steatosis in mice [43].

In agreement with others [44], and the present evidence that Nrf2 deficiency increases NFκB-mediated inflammation, we also show that Nrf2-null mice have exacerbated histologic and biochemical markers of liver steatosis compared to WT mice. Greater liver steatosis in Nrf2-null mice is consistent with a mechanism of enhanced fatty acid uptake by CD36 and greater lipid synthesis mediated by SREBP1c and its downstream lipogenic genes FAS and SCD1 that are upregulated in association with hyperinsulinemia. However, these Nrf2-dependent activities that likely promote hepatic steatosis occurred without affecting hepatic NEFA, which may reflect their rapid esterification to form triglyceride that is mediated by the observed increase in DGAT expression and needed to protect against free fatty acid-induced hepatic lipotoxicity [45].

Consistent with our studies showing GTE-mediated improvements in liver steatosis in association with lowered serum insulin and expression of lipogenic genes [21, 40], the present study shows that GTE reduced liver steatosis regardless of Nrf2 deficiency. Although biochemical measures of hepatic total lipid and triglyceride in Nrf2-null mice fed GTE were lowered compared to Nrf2-null controls, they were not lowered to the extent observed in WT mice fed GTE. This suggests that a modest proportion of the hypolipidemic activities of GTE occur in a Nrf2-dependent manner. The observed lipid-lowering activity is corroborated by our findings that GTE in Nrf2-null mice lowered the expression of CD36 and lipogenic genes (i.e. SREBP1c, FAS, SCD-1) only to the extent observed in WT controls, but not WT mice fed GTE. Although this study was not designed to define events downstream of Nrf2 activation responsible for these hypolipidemic activities, nor could it isolate the anti-inflammatory activities of GTE from its hepatic lipid-lowering activities, Nrf2 maintains energy metabolism by regulating genes for lipid biosynthesis and mitochondrial biogenesis [46] and warrants further investigation. Likewise, consideration for examining Nrf1-dependent hepatoprotection is warranted consistent with Nrf1 transcriptional activities similarly mediated through its binding to ARE to regulate lipid metabolism [47]. However, Nrf1 limits steady-state cellular stress whereas Nrf2 provides secondary defense against unscheduled stress [47], and may have a role in mitigating NASH. Indeed, studies in liver-specific Nrf1 knockout mice show that Nrf1 regulates hepatic lipid metabolism [48] consistent with Nrf1 regulating the expression of PPARα and PPARγ coactivators [49]. Thus, GTE may attenuate NASH through Nrf1 consistent with EGCG reducing liver steatosis in HF-fed mice while upregulating skeletal muscle mRNA expression of Nrf1 and fatty acid oxidation genes [50].

Inflammation contributes to NASH consistent with NASH patients and rodents models of NASH having greater NFκB activation [14, 22, 51]. Although our findings implicate Nrf2 deficiency in increasing NFκB-dependent inflammation, they also suggest that anti-inflammatory activities of GTE on NFκB activation occur independent of Nrf2 responses that suppress ROS. Indeed, HF feeding is known to induce Nrf2 cytoprotective responses that limit ROS accumulation [52]. Thus, GTE may be limited in further upregulating Nrf2-dependent defenses, and supports examining Nrf2-dependent protection by GTE in other NASH models, consistent with the flavone baicalein, a Nrf2 activator, suppressing liver steatosis and inflammation in mice fed a MCD diet [53].

Mechanisms other than Nrf2-mediated alterations in intracellular redox status, such as receptor-mediated pathways, are also known to regulate NFκB activation during NASH [54]. Greater inflammation in NASH patients is associated with small intestinal bacteria overgrowth and greater TLR4 expression on CD14 positive cells that is correlated to plasma IL-8 [31]. Alternatively, studies in TNFR1 knockout mice [32], TNFR1 knockin mice that are unable to down-regulate TNFα-dependent inflammation [55], or treatment with anti-TNFα antibodies [56] support that inhibiting TNFR signaling attenuates NASH. Consistent with these lines of study, we show that hepatic TNFR1 and TLR4 expression that are otherwise increased by Nrf2 deficiency are normalized by GTE in Nrf2-null mice to the extent observed in WT mice fed GTE. Thus, these receptor-mediated pathways warrant consideration to better define anti-inflammatory activities of GTE associated with lowered hepatic TNFα by GTE in obese models of NASH [21, 22] as well as studies showing that EGCG suppresses TLR4 signaling in LPS-stimulated adipocytes [57] and reduces hepatic TLR4 protein in a mouse model of viral hepatitis [58]. Evidence also supports that polyphenols favorably improve the intestinal microbiota composition to mitigate inflammatory-related disorders [59].

In conclusion, GTE protects against HF-induced liver injury and NASH by lowering hepatic lipid accumulation and NFκB-dependent inflammation. Suppression of NFκB-dependent inflammation by GTE is likely independent of Nrf2 signaling and these anti-inflammatory activities may contribute, at least in part, to its hepatic lipid-lowering activities. Identifying the mechanism by which GTE mitigates NFκB-dependent inflammation, such as that mediated through TNFR1 and/or TLR4, warrants further investigation. Furthermore, it is likely that pleotropic effects of GTE may protect against NASH through multiple mechanisms, consistent with EGCG regulating lipid metabolism by activating PPARα and inhibiting PPARγ [60]. Regardless of the mechanism, green tea is among the most commonly used dietary supplements, it is considered safe when ingested as recommended [61], and it or EGCG may provide therapeutic potential in protecting obese individuals from NASH.

Acknowledgments

J Li, TN Sapper, JE Manautou and RS Bruno designed the study; J Li, TN Sapper, E Mah, KE Schill, C Chitchumroonchokchai, MV Moller, JD McDonald, PR Rohrer, S Rudraiah, and RS Bruno conducted experiments and analyzed data; J Li and RS Bruno wrote the manuscript. All authors read, provided substantial intellectual input, and approved the final manuscript.

This work was supported by grants to RSB from USDA-NIFA (2014-67017-21761), The Ohio State University (OSU) Food Innovation Center, the Molecular Carcinogenesis and Chemoprevention Program of the OSU Comprehensive Cancer (National Institutes of Health, National Cancer Institute P30CA16058) and the OSU Center for Advanced Functional Foods Research and Entrepreneurship as well as state and federal funds appropriated to the OSU Ohio Agricultural Research and Development Center.

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Abbreviations

ANOVA	analysis of variance
CD-36	cluster of differentiation-36
DGAT	diglyceride acyltransferase
DMEM	Dulbecco's Modified Eagle Medium
EGCG	epigallocatechin gallate
FAS	fatty acid synthase
GTE	green tea extract
HPRT	hypoxanthine-guanine phosphoribosyl transferase
IKK	IκB kinase
iNOS	inducible nitric oxide synthase
Keap1	Kelch-like ECH-associated protein 1
LDH	lactate dehydrogenase
MCD	methionine choline deficient
MCP-1	monocyte chemoattractant protein-1
MDA	malondialdehyde
NAFLD	nonalcoholic fatty liver disease
NASH	nonalcoholic steatohepatitis
NEFA	nonesterified fatty acid
NFκB	nuclear factor κB
Nqo1, NADPH	quinone oxidoreductase 1
Nrf2	nuclear factor erythroid-2-related factor-2
phospho-p65	phosphorylated-p65
ROS	reactive oxygen species
SCD1	stearoyl-CoA desaturase-1
SREBP-1c	sterol regulatory element binding protein-1c
TBA	thiobarbituric acid
TBST	Tris-buffered saline with Tween-20
TLR4	toll-like receptor-4
TPP	1,1,3,3-tetramethoxypropane
TNFα	tumor necrosis factor-α
TNFR1	tumor necrosis factor receptor-1

WT	wild-type

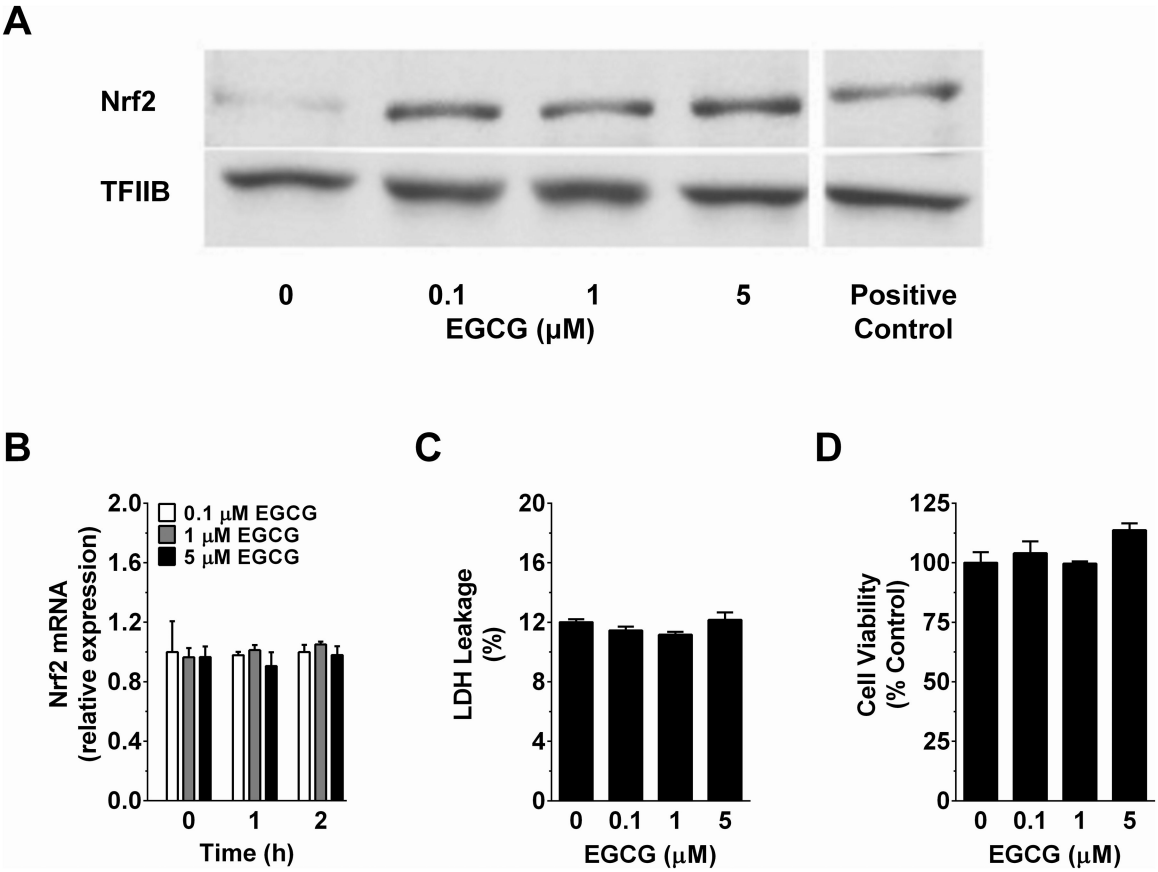

Figure 1. Time- and dose-response effects of EGCG on cell cytotoxicity and nuclear accumulation and mRNA expression of Nrf2 in HC-04 human hepatocytes

HC-04 were treated with 0-5 μM EGCG for up to 2 h as described under Methods. A) Representative Western blot of Nrf2 from isolated nuclear fractions of HC-04 cells following 2 h incubation with EGCG or 100 μM t-butylhydroperoxide (positive control). B) HC-04 cells were treated for up to 2 h with 0.1-5 μM EGCG before isolating total RNA using Trizol and reverse transcribing for RT-PCR analysis of Nrf2 mRNA expression. C) LDH leakage and D) cell viability were assessed using spectrophotometric kits as described under Methods following treatment of HC-04 for 2 h with 0-5 μM EGCG. Shown are mean responses of experiments performed in triplicate. Data were analyzed by 1- or 2-way ANOVA as appropriate. There were no statistically significant time- or concentration-dependent effects of EGCG. Abbreviations: EGCG, epigallocatechin gallate; LDH, lactate dehydrogenase; Nrf2, nuclear factor erythroid-2-related factor-2.

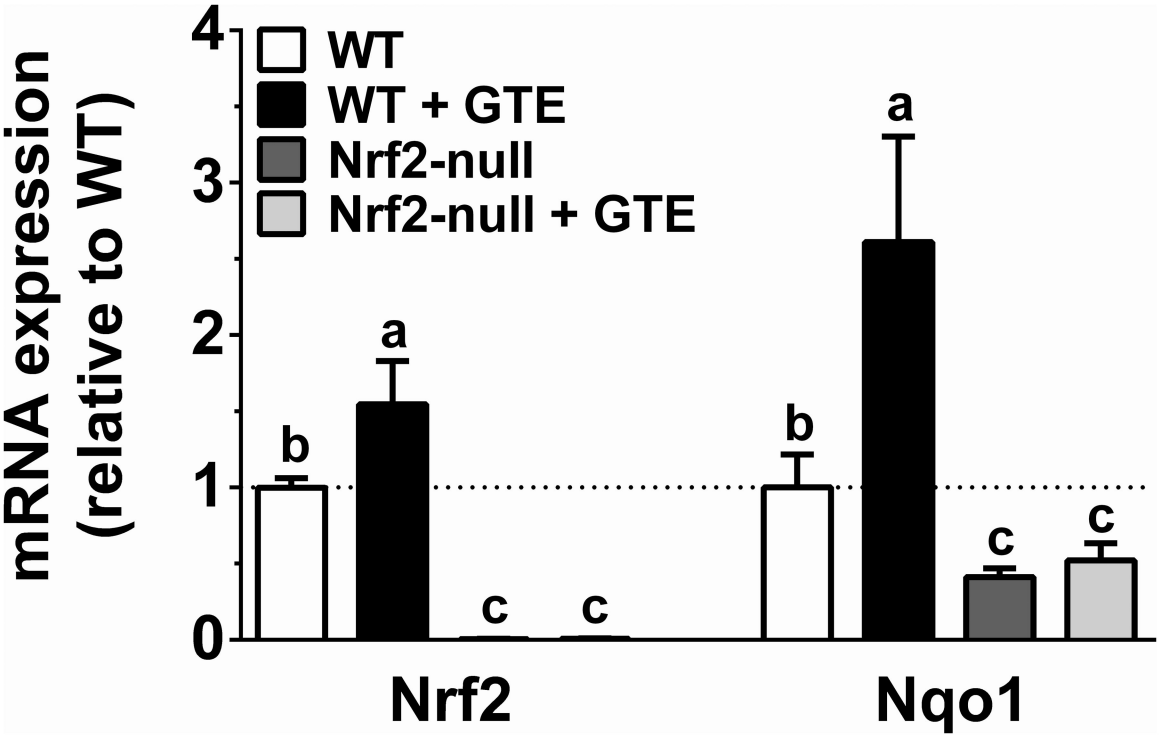

Figure 2. Hepatic mRNA expression of Nrf2 (A) and Nqo1 (B) in Nrf2-null and wild-type mice fed a high-fat diet containing GTE at 0 or 2% for 8 wk

RNA was isolated using Trizol and reverse transcribed for RT-PCR analysis using the primers described in Table 1. Data (means ± SEM, n = 10-12 mice per group) were analyzed by 2-way ANOVA with Newman–Keuls post-test to evaluate main and interactive effects. Groups without a common letter are significantly different ($P<0.05$). Abbreviations: GTE, green tea extract; Nrf2, nuclear factor erythroid-2-related factor-2; Nqo1, NADPH:quinone oxidoreductase 1; WT, wild-type.

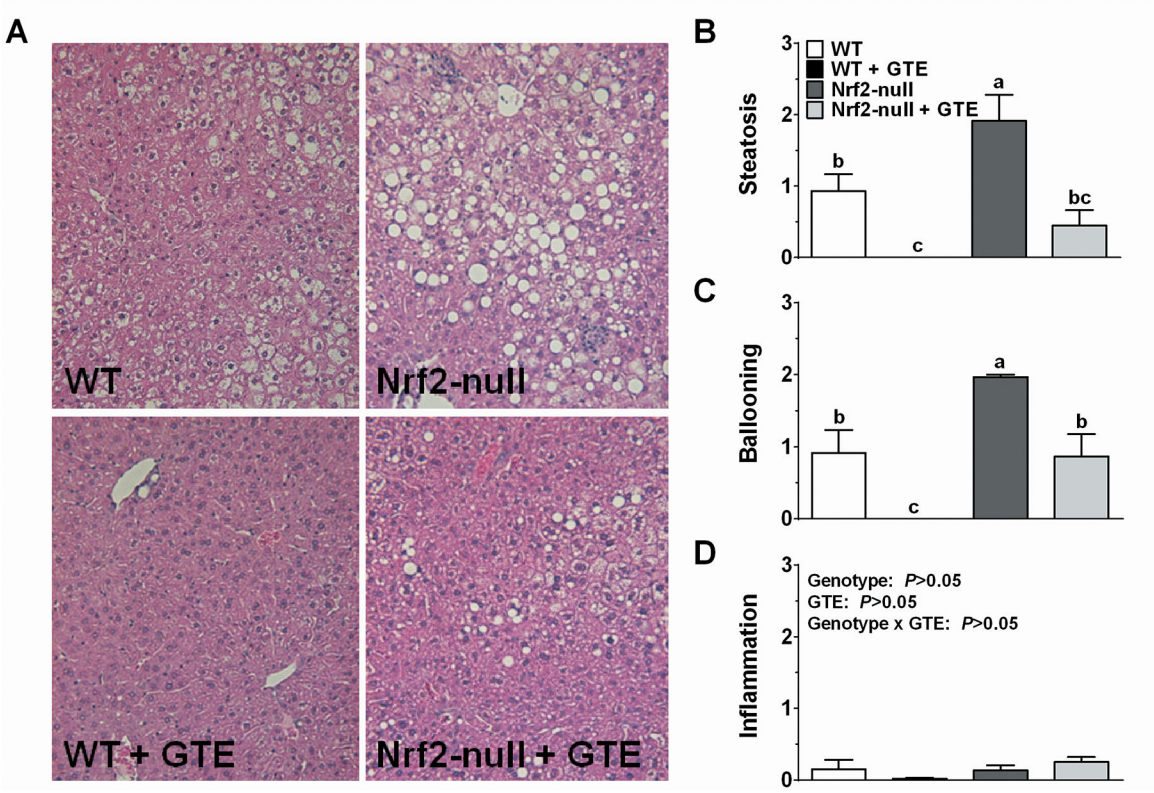

Figure 3. Histologic evaluation of livers from Nrf2-null and wild-type mice fed a high-fat diet containing GTE at 0 or 2% for 8 wk

A) Representative hematoxylin and eosin-stained liver sections (original magnification 200x) in WT and Nrf2-null mice fed a high fat with or without GTE. Nrf2-null mice had exacerbated macrovesicular steatosis and hepatocyte ballooning relative to WT mice. GTE reduced these parameters regardless of genotype, but the effects were more pronounced in WT mice. Histologic scoring of liver steatosis (B), hepatocyte ballooning (C), and inflammatory infiltrates. Data were analyzed by 2-way ANOVA with Newman–Keuls post-test to evaluate main and interactive effects. Groups without a common letter are significantly different ($P<0.05$). Data are means ± SEM, n = 10-12 mice per group). Abbreviations: GTE, green tea extract; Nrf2, nuclear factor erythroid-2-related factor-2; WT, wild-type.

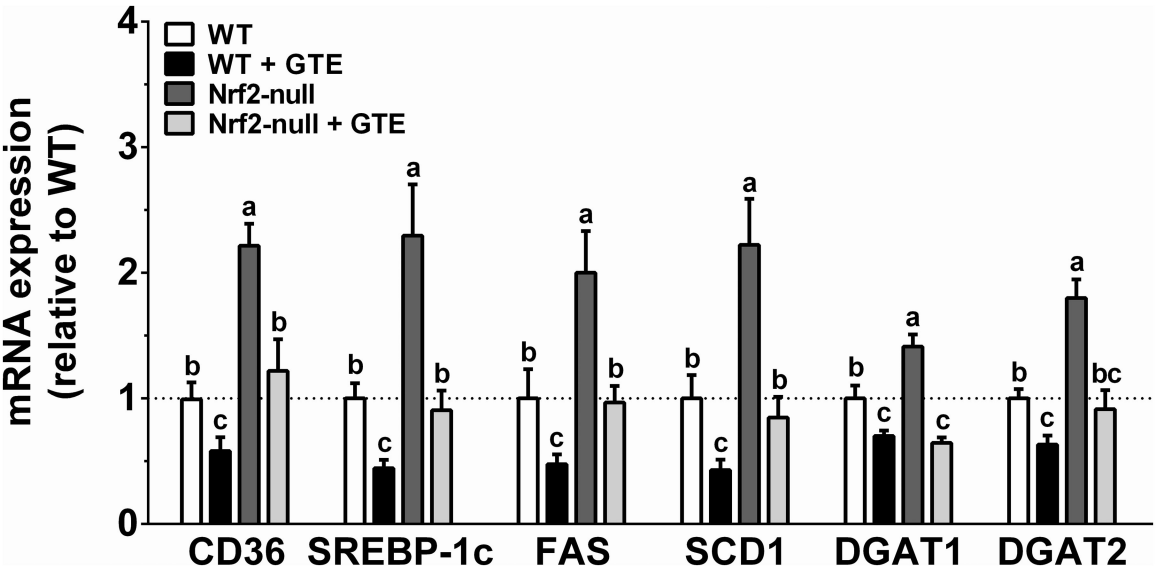

Figure 4. Hepatic mRNA expression of genes involved in free fatty acid uptake and lipid synthesis in Nrf2-null and wild-type mice fed a high-fat diet containing GTE at 0 or 2% for 8 wk

RNA was isolated using Trizol and reverse transcribed for RT-PCR analysis using the primers described in Table 1. Data (means ± SEM, n = 10-12 mice per group) were analyzed by 2-way ANOVA with Newman–Keuls post-test to evaluate main and interactive effects. Groups without a common letter are significantly different ($P<0.05$). Abbreviations: CD36, cluster of differentiation-36; DGAT, diglyceride acyltransferase; FAS, fatty acid synthase; GTE, green tea extract; Nrf2, nuclear factor erythroid-2-related factor-2; SCD1, stearoyl-CoA desaturase-1; SREBP-1c, sterol regulatory element binding protein-1c; WT, wild-type.

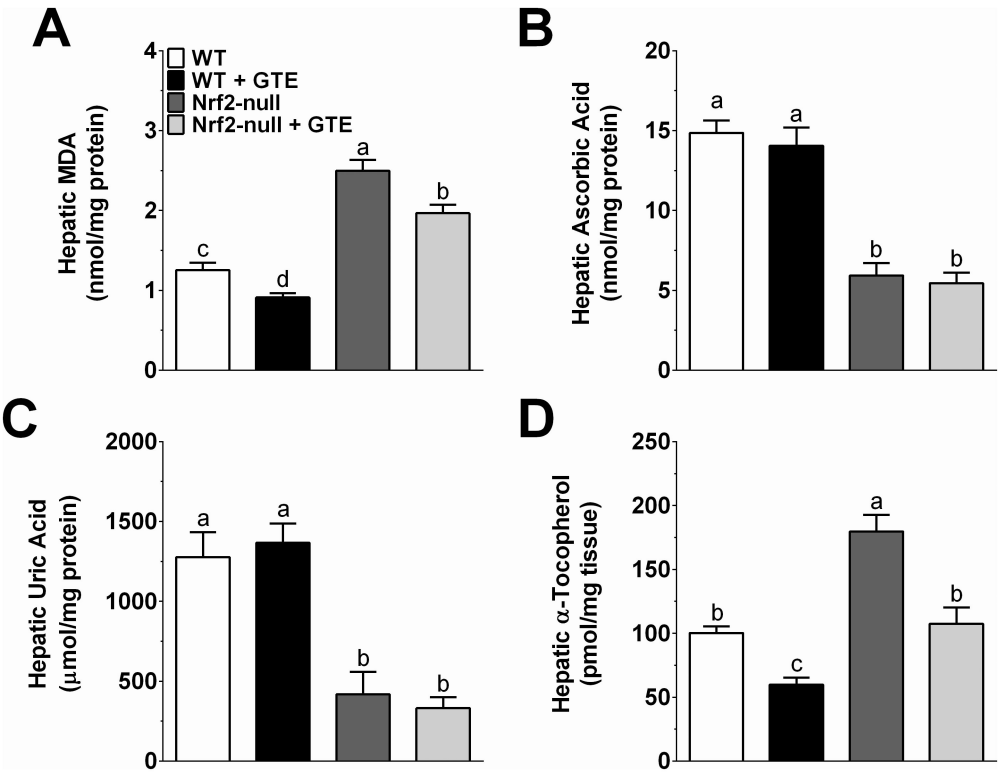

Figure 5. Hepatic lipid peroxidation and antioxidant markers in Nrf2-null and wild-type mice fed a high-fat diet containing GTE at 0 or 2% for 8 wk

A) Hepatic MDA was measured by HPLC-FL following incubation of liver homogenates with thiobarbituric acid and extraction with butanol. B-C) Hepatic ascorbic acid and uric acid were measured by HPLC-ECD following deproteinization of liver homogenates. D) Hepatic α-tocopherol was measured by HPLC-ECD following alcoholic saponification and extraction with hexane. Data (means ± SEM, n = 10-12 mice per group) were analyzed by 2-way ANOVA with Newman-Keuls post-test to evaluate main and interactive effects. Groups without a common letter are significantly different ($P<0.05$). Abbreviations: GTE, green tea extract; MDA, malondialdehyde; Nrf2, nuclear factor erythroid-2-related factor-2; WT, wild-type.

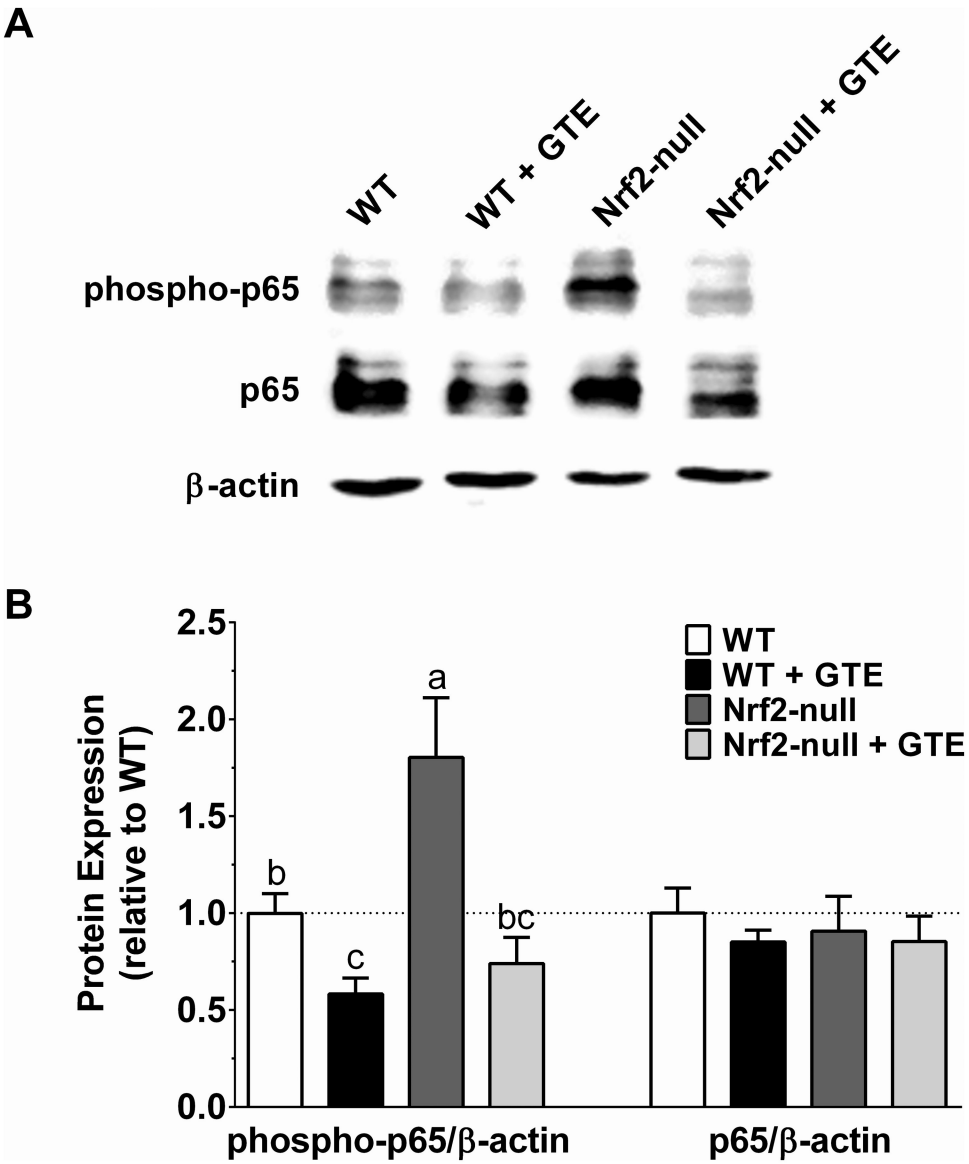

Figure 6. Hepatic protein of the phosphorylated p65 subunit of NFκB or total p65 in Nrf2-null and wild-type mice fed a high-fat diet containing GTE at 0 or 2% for 8 wk

A) Representative Western blot of phospho-p65 and p65 and the loading control β-actin. B) Quantitative densitometry analysis of target protein accumulation in total cellular extracts. Data (means ± SEM, n = 10-12 mice per group) were analyzed by 2-way ANOVA with Newman-Keuls post-test to evaluate main and interactive effects. Groups without a common letter are significantly different ($P<0.05$). Abbreviations: GTE, green tea extract; Nrf2, nuclear factor erythroid-2-related factor-2; phospho-p65; phosphorylated p65 subunit of NFκB; WT, wild-type.

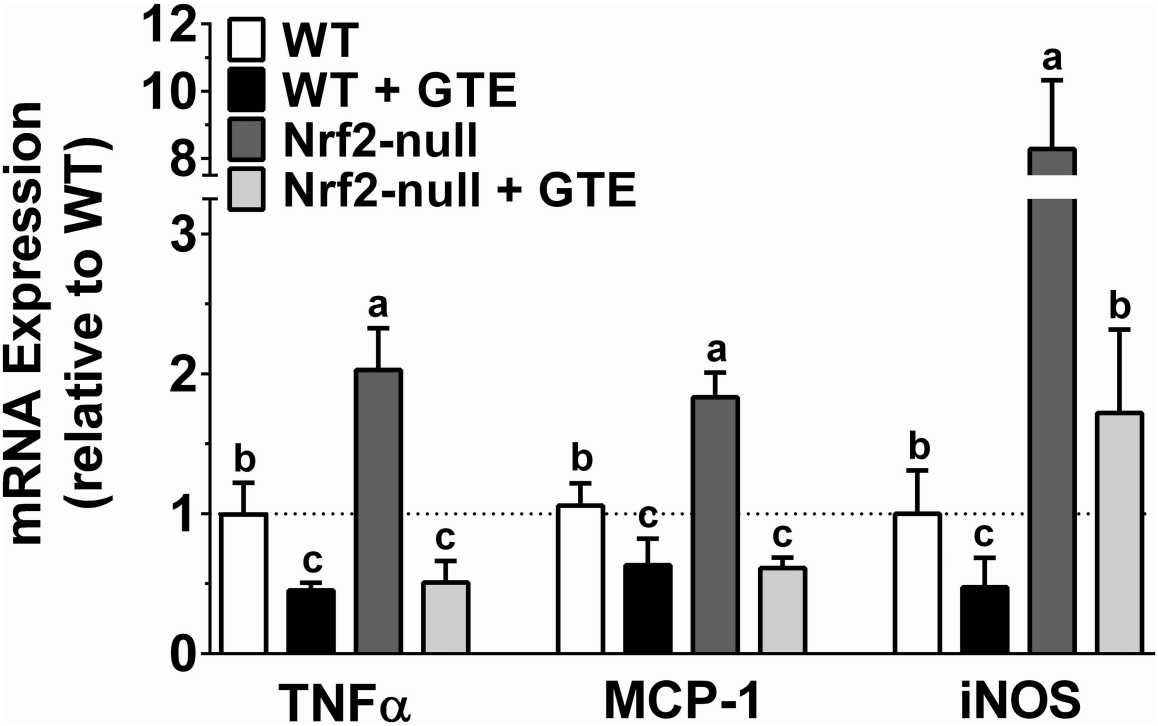

Figure 7. Hepatic mRNA expression of pro-inflammatory genes in Nrf2-null and wild-type mice fed a high-fat diet containing GTE at 0 or 2% for 8 wk

RNA was isolated using Trizol and reverse transcribed for RT-PCR analysis for the NFκB-dependent targets TNFα, MCP-1, and iNOS using the primers described in Table 1. Data (means ± SEM, n = 10-12 mice per group) were analyzed by 2-way ANOVA with Newman-Keuls post-test to evaluate main and interactive effects. Groups without a common letter are significantly different ($P<0.05$). Abbreviations: GTE, green tea extract; iNOS, inducible nitric oxide synthase; MCP-1, monocyte chemoattractant protein-1; Nrf2, nuclear factor erythroid-2-related factor-2; TNFα, tumor necrosis factor-α: phospho-p65; WT, wild-type.

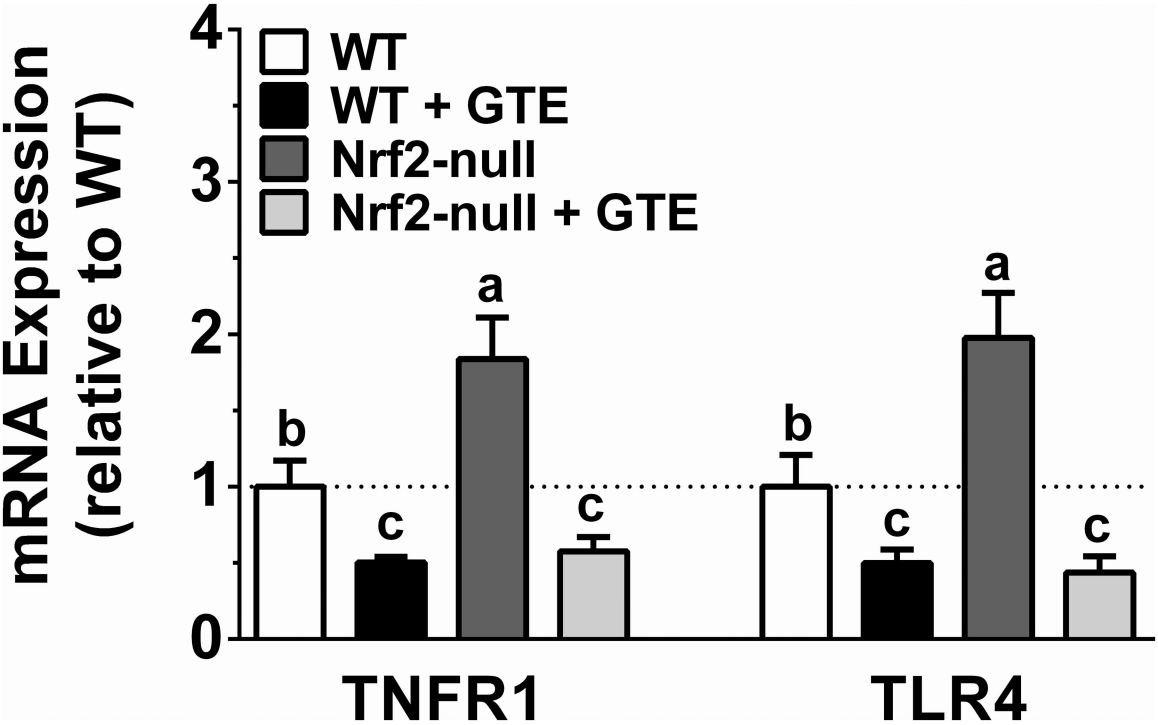

Figure 8. Hepatic mRNA expression of TNFR1 and TLR4 in Nrf2-null and wild-type mice fed a high-fat diet containing GTE at 0 or 2% for 8 wk

RNA was isolated using Trizol and reverse transcribed for RT-PCR analysis for TNFR1 and TLR4 using the primers described in Table 1. Data (means ± SEM, n = 10-12 mice per group) were analyzed by 2-way ANOVA with Newman-Keuls post-test to evaluate main and interactive effects. Groups without a common letter are significantly different ($P<0.05$). Abbreviations: GTE, green tea extract; Nrf2, nuclear factor erythroid-2-related factor-2; TLR4, toll-like receptor 4; TNFR1, tumor necrosis factor receptor-1; WT, wild-type.

Table 1

Primers used for RT-PCR gene expression studies[a,b]

Gene	Accession Number	Forward Primer	Reverse Primer
β-actin*	NM_001101	CCATCGAGCACGGCATC	ATTGTAGAAGGTGTGGTGCAGA
CD36	NM_007643	CCGAGGACCACACTGTGTC	AACCCCACAAGAGTTCTTTCAAA
DGAT1	NM_010046	TCCGCCTCTGGGCATTC	GAATCGGCCCACAATCCA
DGAT2	NM_026384	AGTGGCAATGCTATCATCATCGT	AAGGAATAAGTGGGAACCAGATCA
FAS	NM_007988	GGAGGTGGTGATAGCCGGTAT	TGGGTAATCCATAGAGCCCAG
HPRT	NM_013556	CACAGGACTAGAACACCTGC	GCTGGTGAAAAGGACCTCT
iNOS	NM_010927	TTCTGTGCTGTCCCAGTGAG	TGAAGAAAACCCCTTGTGCT
MCP-1	NM_011333	TGATCCCAATGAGTAGGCTGGAG	ATGTCTGGACCCATTCCTTCTTG
Nrf2*	NM_006164	GCTCATACTCTTTCCGTCGC	ATCATGATGGACTTGGAGCTG
Nrf2	NM_010902	CGAGATATACGCAGGAGAGGTAAGA	GCTCGACAATGTTCTCCAGCTT
Nqo1	NM_008706	TTCTGTGGCTTCCAGGTCTT	AGGCTGCTTGGAGCAAAATA
SCD1	NM_009127	TTCTTGCGATACACTCTGGTGC	CGGGATTGAATGTTCTTGTCGT
SREBP-1c	NM_011480	GCAGCCACCATCTAGCCTG	CAGCAGTGAGTCTGCCTTGAT
TLR4	NM_021297	CCTCTGCCTTCACTACAGAGACTTT	TGTGGAAGCCTTCCTGGATG
TNFα	NM_013693	CTCCAGGCGGTGCCTATG	GGGCCATAGAACTGATGAGAGG
TNFR1	NM_011609	GGGCACCTTTACGGCTTCC	GGTTCTCCTTACAGCCACACA

[a] All primer sequences are for mice except for human sequences that are annotated by an asterisk.

[b] Abbreviations: CD36, cluster of differentiation-36; DGAT, diglyceride acyltransferase; FAS, fatty acid synthase; HPRT, hypoxanthine-guanine phosphoribosyl transferase; iNOS, inducible nitric oxide synthase; MCP-1, monocyte chemoattractant protein-1; Nrf2, nuclear factor erythroid-2-related factor-2; Nqo1, NAD(P)H dehydrogenase:quinone 1; SCD1, stearoyl-CoA desaturase-1; SREBP-1c, sterol regulatory element binding protein-1c; TLR4, toll-like receptor-4; TNFα, tumor necrosis factor-α; TNFR1, tumor necrosis factor receptor-1

Table 2

Body composition, food intake, and serum and liver metabolic parameters in Nrf2-null and wild-type mice fed a high-fat diet containing GTE at 0% or 2% for 8 wk[a,b,c]

	WT	WT + GTE	Nrf2-null	Nrf2-null + GTE
Initial Body Weight (g)	26.9 ± 0.4	27.0 ± 0.5	28.4 ± 0.8	28.9 ± 0.6
Final Body Weight (g)	43.1 ± 0.9^{a}	29.5 ± 1.1^{c}	38.8 ± 1.1^{b}	32.0 ± 1.3^{c}
Food Intake (g/d)	2.9 ± 0.1	2.5 ± 0.3	2.3 ± 0.1	2.4 ± 0.4
Liver Mass (g)	1.40 ± 0.1^{a}	1.05 ± 0.0^{bc}	1.16 ± 0.1^{ab}	0.87 ± 0.1^{c}
Total Adipose Tissue (g)	3.93 ± 0.2^{a}	1.03 ± 0.2^{c}	3.34 ± 0.1^{a}	2.12 ± 0.3^{b}
Hepatic Total Lipid (mg/g liver)	231.7 ± 16.1^{b}	156.9 ± 13.4^{c}	312.5 ± 19.0^{a}	219.2 ± 17.9^{b}
Hepatic Triglyceride (μmol/g liver)	72.9 ± 10.5^{b}	34.4 ± 4.0^{c}	122.7 ± 13.4^{a}	71.1 ± 10.5^{b}
Hepatic Cholesterol (μmol/g liver)	8.4 ± 0.3^{b}	8.5 ± 0.4^{b}	12.3 ± 0.8^{a}	10.9 ± 1.4^{a}
Hepatic NEFA (μmol/g liver)	34.5 ± 28.3	35.4 ± 5.4	31.5 ± 3.9	34.4 ± 2.2
Serum ALT (U/L)	28.5 ± 1.6^{b}	11.0 ± 0.6^{c}	85.8 ± 13.9^{a}	23.5 ± 6.9^{bc}
Serum Glucose (mmol/L)	17.6 ± 1.2^{a}	13.0 ± 0.9^{b}	15.7 ± 0.6^{a}	11.4 ± 1.2^{b}
Serum Insulin (pmol/L)	90.2 ± 8.1^{b}	52.9 ± 7.4^{c}	123.7 ± 9.2^{a}	63.8 ± 11.4^{c}
Serum NEFA (mmol/L)	0.94 ± 0.0^{a}	0.68 ± 0.0^{b}	0.93 ± 0.1^{a}	0.93 ± 0.0^{a}

[a]Total adipose mass represents the sum of epididymal and retroperitoneal fat pads.

[b]Data are means ± SEM, n = 10-12 in each group. Data were analyzed by 2-way ANOVA with Newman-Keuls post-test to assess group mean differences. Means in a row not sharing a common letter are significantly different, $P \leq 0.05$.

[c]Abbreviations: ALT, alanine aminotransferase; GTE, green tea extract; HF, NEFA, non-esterified fatty acids; Nrf2, nuclear factor erythroid-2-related factor-2; WT, wild-type.